

Expression of p16 in sporadic primary uveal melanoma^{★☉}

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Expression of p16 protein, intragenic mutations of CDKN2A and hypermethylation of CDKN2A promoter region in 41 sporadic primary uveal melanomas were studied. There were 2 cases of spindle cell B histological type, 11 of A + B and 28 of mixed type. All melanomas infiltrated sclera but in 28 cases infiltration was superficial while in 13 profound. In 7 cases the tumor infiltrated the optic nerve. Expression of p16 was studied by immunohistochemistry and recorded by assessment of the proportion of positive tumor cells and staining intensity. Results were expressed as staining index (IRS). Intragenic mutations were studied by PCR-SSCP followed by sequencing, while hypermethylation of the promoter region by CpG methylation assay. In 15% of cases less than 10% of melanoma cells were p16 positive, in 70% of cases less than 50% of cells, while in 7% more than 80% of cells stained for p16 (mean IRS for all cases was 4.87 ± 2.43). In B type the IRS was 8.5 ± 0.7 , in A + B type 6.0 ± 2.1 and in the mixed type 4.17 ± 2.43 (differences statistically significant). In melanomas profoundly infiltrating sclera mean IRS was 4.16, while in those infiltrating optic nerve 3.71 (statistically not significant). Analysis of the intragenic mutations revealed in two patients a GAC/GAT substitution in codon 84 – a silent mutation. No hypermethylation of the CpG island of the p16 promoter region was found. In conclusion, we found that the degree of p16 expression is related to the histological type of tumor but not to the histological indicators of tumor invasiveness and that intragenic mutations and promoter hypermethylation are not major mechanisms of p16 inactivation in sporadic uveal melanoma.

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Thirteen percent of primary melanomas develop within an eye and the eye is, next to skin, the most frequent site of melanoma localization (30–79%). Despite the fact that both tumors arise from melanocytes their biology differs substantially. Some differences in the clinical course of the disease might be assigned to anatomic localization or immune response to the primary tumor. Nevertheless, the kinetics of disease development or significant variation of metastases responsiveness to chemical agents could also be due to other factors such as genetic alterations. Similarly to skin melanoma various stages of the eye melanoma progression may be distinguished (Garner & Klintworth, 1994). The progression of skin melanoma is associated with inactivation of a number of tumor suppressor genes (Fountain *et al.*, 1990). These processes, however, have not been extensively studied in eye melanoma so far. Merbs & Sidransky (1999) reported an analysis of a tumor suppressor gene an inhibitor of cyclin-dependent kinase (CDK) 4 and 6 – p16 Ink4a (CDKN2A/MTS-1) locus – in 33 primary sporadic uveal melanomas for loss of heterozygosity (LOH) of chromosome 9p21 and examined the mechanisms of p16 inactivation in these tumors.

The CDKN2A gene was mapped to the 9p21 chromosome region. It encodes the cyclin dependent kinase inhibitor p16 and p14 ARF, an alternatively spliced form (Kamijo *et al.*, 1997). Both proteins act as negative cell cycle regulators. Mutations and other genetic alterations in CDKN2A were found in cell lines derived from skin melanoma but role of these changes in initiation and progression of skin melanoma is still under debate. Studies of genetic changes in CDKN2A and the expression level of p16 protein suggest that loss of heterozygosity and protein expression play important roles in the progression of melanoma (Keller-Melchior *et al.*, 1998) and are characteristic for the advanced stages of this type of tumor (Cairns *et al.*, 1995).

In this work p16 expression was examined in various histological types of uveal melanoma

and in cases displaying signs of invasiveness such as profound sclera or optic nerve infiltration. In addition we analyzed p16 intragenic mutations and hypermethylation of the promoter region as possible mechanisms of inactivation of this gene. Studies were carried out in fresh non cultured cells and in paraffin embedded tissues.

MATERIALS AND METHODS

Patients. Enucleation due to eye melanoma was performed in 41 patients (mean age 56 years; range 21–77). In 37 patients the melanoma was localized within the uvea while in 4 within the uvea and ciliary body. Following enucleation tumor tissue was divided into two parts. One was fixed in formalin for histological diagnosis and immunohistochemistry, and the other was frozen in liquid nitrogen for further DNA isolation. Histological diagnosis of uveal melanoma assessed for cell type using the modified Callender system (McLean *et al.*, 1983) revealed: 2 cases of spindle B, 11 cases of spindle A + B, and 28 of mixed type. All melanomas infiltrated sclera, however, in 28 cases infiltration was superficial while in 13 profound. In 7 cases tumors infiltrated the optic nerve.

Immunohistochemistry. Expression of p16 proteins in melanoma tissue was analyzed immunohistochemically by the labelled streptavidin/biotin method on formalin-fixed, paraffin embedded tissues. Sections of 4 µm were dewaxed in xylene, and rehydrated through descending graded alcohols to phosphate-buffered saline, pH 7.4 (NaCl/P_i). Due to a very high melanine content slides were incubated for 30 min in 0.25% aqueous potassium permanganate solution, washed in tap water and soaked in 5% aqueous solution of oxalic acid for 2–5 min (until sections become clear). Then, following blocking of endogenous peroxidase activity with 1% H₂O₂ (30 min) and non-specific antibody binding with 1% bo-

vine albumin in NaCl/P_i for 30 min, sections were incubated with primary antibodies. Incubation was carried out with polyclonal rabbit anti-human p16 antibodies (Santa Cruz Biotechnology, CA, U.S.A.) in 1:1500 dilution in NaCl/P_i overnight at 4°C followed by 1 h incubation at room temperature. Sections were subsequently incubated with prediluted biotinylated link antibody (DAKO LSAB 2 Kit, Dako Corp. CA, U.S.A.) for 30 min followed by streptavidin-horseradish peroxidase conjugate one (DAKO LSAB 2 Kit, Dako Corp. CA, U.S.A.) for 30 min. After washing, peroxidase activity was detected using 3,3'-diaminobenzidine as chromogen with H₂O₂ as substrate. The sections were counter stained with haematoxylin, dehydrated, cleared in xylene and mounted.

For recording of p16 immunohistochemical staining a semi-quantitative and subjective grading, considering both the proportion of tumor cells showing positive reaction and intensity of staining was used. The proportion of stained cells (PP) was graded as: 0 – no tumor cells positive, 1 – positive staining in < 10% of tumor cells, 2 – positive staining in 10–50% of tumor cells, 3 – positive staining in 50–80% of tumor cells, 4 – positive staining in > 80% tumor cells. Staining intensity (SI) was graded as: 0 – no staining, 1 – weak staining, 2 – moderate staining, 3 – strong staining. Staining index (IRS): PP times SI (range: 0–12).

DNA extraction. Genomic DNA was extracted from melanoma tissue samples previously frozen in liquid nitrogen. Samples were homogenized and DNA was prepared using a standard method with proteinase K, several phenol/chloroform extractions followed by ethanol precipitation. In samples containing a high amount of melanin DNA was purified using Wizard purification resin (Promega, WI, U.S.A.) or spin columns (Qiagen, CA, U.S.A.) according to the manufacturer's instructions.

PCR-SSCP analysis. For analysis of point mutations in the p16 gene the PCR-SSCP method was used with the following pairs of primers: 1/16F: GGGAGCAGCATGGAGC-

CG; 1/16R: AGTCGCCCCGCCATCCCCT; 2A/16F: AGCTTCCTTTCCGTCATG; 2A/16R: GCAGCACCACCAGCGTG; 2B/16F: AGC-CCAAGTGCAGCGAC; 2B/16R: CCAGGTC-CACCGGCAGA; 2C/16F: TGGACGTGCGC-GATGC; 2C/16R: GGAAGCTCTCAGGGTAC-AAATTC; 3/16F: CCGGTAGGGACGGCAA-GAGA; 3/16R: CTGTAGGACCCTCGGTGACTGATGA. The primers used for PCR-SSCP were end labelled by [γ -³²P]ATP (3000 Ci/mmol, Amersham). PCR was carried out in a total volume of 5 μ l with the following reagents: 1 x PCR buffer, 1.5 mM MgCl₂, dNTPs 0.2 mM each, primers 1 μ M each, 6% Me₂SO, Taq Pol. 2.5U/100 μ l (Promega WI, U.S.A.) and 50 ng of genomic DNA. The amplification program consisted of 35 cycles: 94°C/5'; 94°C/30", 55°C/30", 72°C/30"; 72°C/5' for p16 fragments 2A, 2B and 2C; 94°C/5'; 94°C/30", 58°C/30", 72°C/30"; 72°C/5' for fragments including exons 1 and 3. The PCR products were mixed with 1/9 volume of formamide dye mixture, heated at 95°C/5' and loaded onto the gel. SSCP analysis was carried out in 5% polyacrylamide gel with 10% glycerol, at room temperature at 15 W. The gel was transferred into Whatman 3MM paper, dried and autoradiographed.

Sequencing. Bands showing mobility shift were cut from the gel, eluted into water and reamplified. PCR products were subjected to electrophoresis in 1.5% agarose gels, cut out and purified using gel extraction kit (Qiagen, CA, U.S.A.). The purified PCR products served as template in the cycle sequencing procedure using the fmol DNA sequencing System (Promega, WI, U.S.A.). End labelled amplimers were used as sequencing primers. Products of sequencing reactions were run in a gel containing 7.5 M urea, dried and autoradiographed.

Methylation assay. DNA (5 μ g) was modified using 3 M sodium bisulfite with 10 mM hydroquinone according to the previously described method (Herman *et al.*, 1996) and CpG Methylations Assay (Oncor, MD, U.S.A.). Modified DNAs were purified by Wizard puri-

fication resin (Promega, WI, U.S.A.) and eluted into water. The reaction was terminated by NaOH treatment, followed by ethanol precipitation. DNA was resuspended in water and used in PCR reaction with primers described previously (Herman *et al.*, 1996). PCR reactions were performed in 10 μ l volume with Gold Taq Polymerase (Perkin Elmer, NJ, U.S.A.) and consisted of the following steps: 95°C/10'; 95°C/45'', 60°C/45'', 72°C/60'' \times 35 and 72°C/5' of terminal elongation. PCR products were analyzed by electrophoresis in a 2% agarose gel.

Statistics. Statistical analysis was carried out using Student's *t*-test χ^2 and Spearman rank correlation tests.

RESULTS

Immunohistochemical evaluation of p16 in uveal melanoma tissue

Most of p16 positive melanoma cells showed staining only in nuclei. In a few cases staining in both nuclei and the cytoplasm was observed (Fig. 1). In one case no p16 staining was detected (PP – 0). In 6 cases < 10% positive cells (PP – 1), in 22 cases 10–50% positive cells (PP

– 2), in 9 cases 50–80% positive cells (PP – 3) and in 3 cases > 80% positive cells (PP – 4) were found. Staining index negative or very weak (IRS \leq 2) was demonstrated by 9 cases (23%), very strong index (IRS 8–9) 7 cases (17%) and intermediate (IRS 3 – 6) 25 cases (61%). Mean IRS for all cases was 4.87 ± 2.43 . In spindle cell type B the mean p16 IRS value was 8.5 ± 0.7 , in A + B type 6.0 ± 2.1 and in the mixed type 4.17 ± 2.24 . Statistically significant differences between all histological types ($P < 0.002$ – 0.006) with the exception between type B and type A + B were found.

In uveal melanomas profoundly infiltrating sclera the mean p16 IRS value was 4.16 ± 1.85 , while in those infiltrating optic nerve 3.71 ± 1.88 . No statistical differences (using Student's *t*-test) between melanomas superficially and profoundly infiltrating sclera ($P < 0.09$) or optic nerve ($P < 0.76$) were found (Table 1).

PCR-SSCP analysis

In two patients a mobility shift different in a fragment of exon 2 of the p16 gene was observed. Sequencing analysis of these bands revealed a substitution in codon 84 GAC/GAT (Asp/Asp – silent mutation) (Fig. 2).

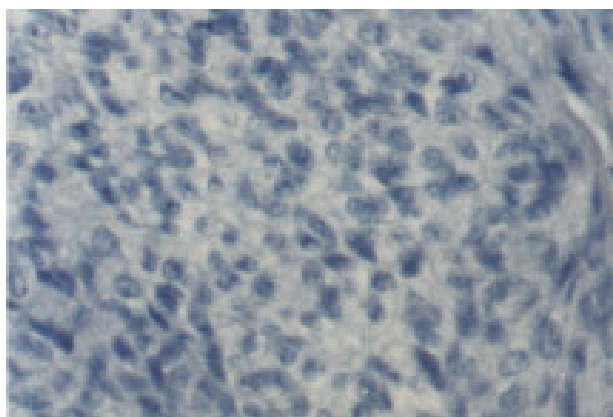
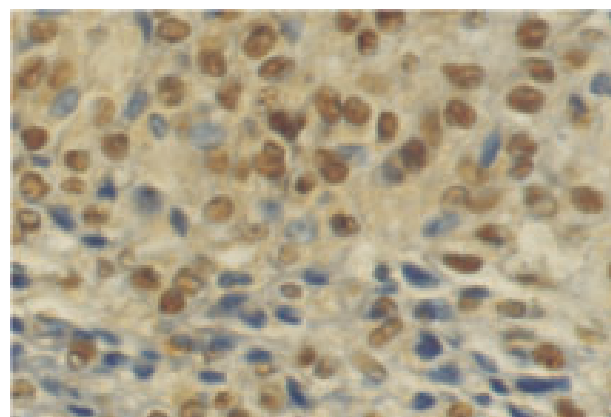
A**B**

Figure 1. Immunohistochemical staining of p16 protein in uveal melanoma.

A, control – no p16 antibody. B, nuclear and nuclear/cytoplasmic localization of p16 protein.

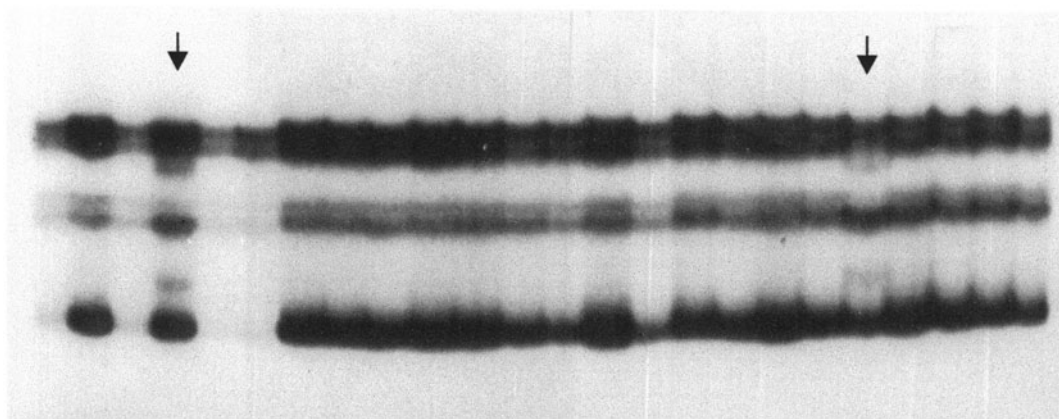


Figure 2. Example of PCR-SSCP analysis of p16 gene – fragment of exon 2.

The arrows indicate lanes with mutated bands.

Table 1. p16 IRS index in different histological type of uveal melanoma

p16 IRS	B	A+B	mix
0-2	0 (0%)	1 (9%)	8 (29%)
3-6	0 (0%)	7 (69%)	18 (64%)
7-9	2 (100%)	3 (27%)	2 (7%)

Methylation analysis

The CpG islands of the p16 promoter regions demonstrated PCR products only with primers designed for unmethylated DNA. We observed no PCR products in the reaction with primers for methylated DNA with the exception of control DNA used as a control of modification and PCR correctness (Fig. 3).

DISCUSSION

There are two major findings of the study: (i) the degree of p16 expression is statistically related to histological type of the primary sporadic melanoma but is not a histological indicator of tumor invasiveness, (ii) intragenic mutations or promoter hypermethylation are not major mechanisms of p16 inactivation in sporadic primary uveal melanoma.

The p16 protein immunostain localizes dominantly to the nucleus, while cytoplasmic

colocalization might be also seen (Hara *et al.*, 1996). In uveal melanoma cells we observed p16 nuclear immunostaining and in a few cases cytoplasm co-immunolocalization. A significant heterogeneity in the percentage of p16 positive cells and staining intensity was observed. Only in one case there were no positive cells present. About 15% of cases demonstrated p16 expression in less than 10% of cells, while about 70% of cases in less than 50%. Only 7% of uveal melanomas showed more than 80% of cells positively stained for p16. In order to evaluate p16 expression more precisely the staining index (IRS) which is based on number of positive cells and staining intensity was adopted (Remmele & Stegner, 1987). Statistically significant differences of p16 IRS were observed between histological types of the tumor. The lowest IRS was found in the mixed type uveal melanoma. Lower IRS than in the whole material studied was also found in tumors displaying signs of invasiveness. However, probably due to the small number of cases in the analysis, the differences were statistically not significant. There is only one report on p16 expression in uveal melanoma so far (Coupland *et al.*, 1998). In these studies p16 showed both nuclear and cytoplasmic staining. Only in 45% of cases positive p16 staining was observed. The amount of positively stained cells varied between 5–25% (mean 12%) of tumor cells. No correlation between histological type and p16

expression was seen. Those results significantly differ from the data presented in this study. One of the possible reasons of the discrepancy might be the difference in the antibody used. Coupland *et al.* (1998) employed monoclonal antibodies, while in our studies polyclonal antibodies were used. In skin melanoma Reed *et al.* (1995) demonstrated that p16 retains its expression in melanoma *in situ* and in the majority of primary melanomas, while 52% of primary invasive tumors and

tases of uveal melanoma were not analyzed in this study. However, the number of negative or weakly positive p16 primary sporadic uveal melanomas was significantly lower than the corresponding number of primary skin melanomas suggesting some differences in genetic mechanisms of progression between these tumors.

There is a number of possible mechanisms of p16 inactivation in melanoma and uveal melanoma. They include homozygous deletion

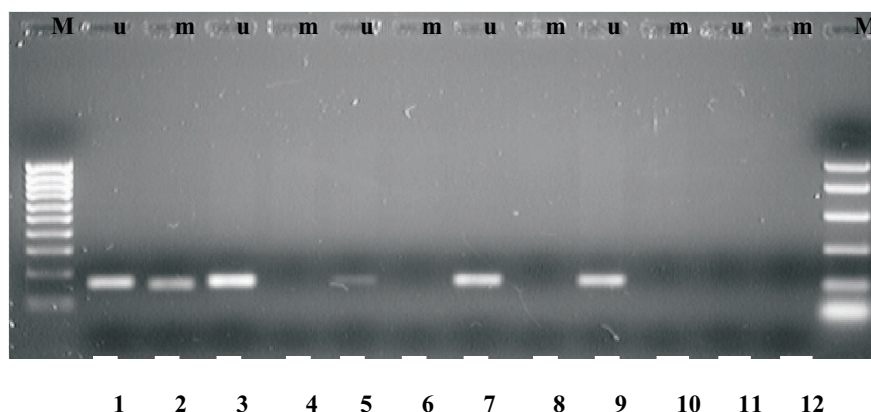


Figure 3. Results of methylation analysis of CpG islands in the promoter region of the p16 gene on 1.5% agarose gel.

Primer sets were designed as unmethylated (u) and methylated (m). In lanes marked M, DNA size marker (left: GeneRuler 100 bp DNA ladder, right: PCR Marker Promega); lanes 1 and 2, products of MSP analysis on control DNA samples; lanes 3–11, examples of MSP analysis on DNA from cancer cells.

72% of metastatic lesions demonstrate partial or complete loss of p16 expression. Similar results were obtained by Grover *et al.* (1998) who showed high p16 expression in benign melanocytic nevi (96% positive) and significant reduction in primary melanomas (31% negative) and metastatic lesions (63% negative). Others correlated loss of p16 expression with tumor cell proliferation and the invasive stage of melanoma (Sparrow *et al.*, 1998). Those data suggest that loss of p16 expression is not necessary for tumor initiation in malignant skin melanoma but is potentially more related to invasiveness or the ability to metastasize. Finally, an association was found between absent or very weak p16 staining and markedly decreased survival rates (Strume & Akslen, 1997). As already pointed out metas-

(Peng *et al.*, 1995), LOH (Flores *et al.*, 1996), intragenic mutations (Smith-Sorensen & Hoving, 1996), hypermethylation of CpG islands of promoter region (Herman *et al.*, 1995) and microsatellite instability (Matsumura *et al.*, 1998). In this study we analyzed two of the possible mechanisms listed above, namely intragenic mutations and methylation of CpG islands in promoter regions. Mutations in the p16 gene are found in melanoma cell lines (Liu *et al.*, 1995). Germinal mutations in this gene have also been reported in familial cutaneous melanoma (Holuska & Hodi, 1998). Somatic mutations are present but with lower frequency. Since small deletions and nonsense mutations are found in the p16 gene which often leads to the loss of the p16 protein product, we have analyzed

p16 gene for the presence of such small genetic changes using the PCR-SSCP. In the p16 gene the substitution C/T was found in two cases, but this mutation had no effect on the amino-acid residue. This silent mutation within p16 were found frequently in other types of cancer patients in Poland, such as lung cancer (unpublished). Accordingly, we regard it as the most frequent p16 polymorphism in this country. Others did not find mutations in the p16 gene in primary sporadic uveal melanoma either (Merbs & Sidranski, 1999).

Next we analyzed possible silencing of p16 gene by hypermethylation of CpG islands in promoter regions – a second possible way of p16 gene inactivation in melanoma. As a result we did not find methylation in the promoter region of this gene. Merbs & Sidranski (1999) observed hypermethylation of p16 promoter region in only 2 of 33 primary uveal melanomas studied. Accordingly, the above results and our study suggest that hypermethylation of the promoter region does not play a major role in p16 inactivation in this type of tumor.

In recent reports LOH at 9p21 and 9p21-22 were found by means of microsatellite marker in 24% (Merbs & Sidranski, 1999) and 32% of sporadic primary uveal melanoma cases, respectively (Ohta *et al.*, 1996). Similarly, LOH at 9p21 is frequently found in sporadic skin melanomas. Furthermore, studies of LOH at 9p21 in primary skin melanoma and melanoma metastases revealed that LOH appeared in the later stages of melanoma development (Morita *et al.*, 1998). It is, however, interesting that in the studies where LOH was reported very limited or no point mutations were found (Ruiz *et al.*, 1998). Moreover, in the study of LOH at 9p21 in uveal melanoma expression of p16 protein was not analyzed. It is, therefore difficult to speculate on the relationship between LOH at 9p21 and p16 expression. On the other hand, a study on sporadic melanoma demonstrated that partial or complete loss of p16 expression is frequently

associated with 9p21 LOH (Funk *et al.*, 1998). Also homozygous p16 losses have been reported in skin melanomas (Wagner *et al.*, 1998).

The differences in p16 expression could be also caused by other mechanisms. Examples include selective loss of the gene locus, failure of other steps in protein biosynthesis or changes in other genes such as Rb, which was reported to be frequently phosphorylated at serine 807 and serine 811, and cyclin D1 was expressed in many of the tumors. Phosphorylation of the COOH terminal region is also a possible mechanism of Rb inactivation in uveal melanomas (Brantly & Harbour, 2000). Also other inhibitors of the cell cycle machinery such as p21, p27, p53 and Ki67 (Mib-1) were studied in uveal melanoma, and an attempt was made to correlate with clinicopathologic parameters. The results suggested, that only p21 and p27 may be involved in tumorigenesis in choroidal melanoma (Brantly & Harbour, 2000). Undoubtedly, studies of the more advanced stages of uveal melanoma cases might help to resolve the genetic cascade involved in uveal melanoma progression and appreciate the role of the p16 gene in this type of cancer.

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